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Antimicrobial activity of hop extracts against *Listeria monocytogenes* in media and in food

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Abstract

Growth of *Listeria monocytogenes* was inhibited in culture media and in certain foods by four hop extracts (I–IV) containing varying concentrations of α- and β-acids. Extracts (II and III) containing the highest concentrations of β-acids were inhibitory at 0.01 mg/l in trypticase soy broth. In food, these hop extracts showed varying magnitudes of inhibition. In coleslaw, hop extract III at 1 mg/g enhanced the rate of inactivation of *L. monocytogenes* Scott A. Hop extract II was inhibitory at 0.1 and 1 mg/ml in skim and 2% milk, and was inhibitory at 1 mg/ml in whole milk. Hop extract II was listericidal in cottage cheese at 0.1 to 3 g/kg. No inhibition of *L. monocytogenes* by hop extract III was observed in Camembert cheese. Overall, the antimicrobial activity of hop extracts in food appeared to increase with acidity and lower fat content. Our results indicate that hop extracts could be used to control *L. monocytogenes* in minimally processed food with low fat content.

Keywords: Hops; β-acids; Lupulone; Isohumulone; Food preservation; Antimicrobial; *Listeria monocytogenes*

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1. Introduction

The hop plant belongs to the botanical family of the Cannabinaceae (Verzele, 1986; Verzele and de Keukeleire, 1991), which includes the genera *Humulus* and *Cannabis*. Cultivated species of hops (*Humulus lupulus*) have anecdotably been used as an ingredient in beer since ancient times (Hoffman, 1956). Hops became an ingredient in beer probably because of its bacteriostatic activity which enhanced quality and stability (Russell and Stewart, 1995). Hops were used for medicinal purposes in cloisters and monasteries in the 8th and 9th centuries (Verzele and de Keukeleire, 1991) and have pharmacological properties (Steidle, 1931).

The blossoms (cones) of the female hop plant contain resins and essential oils which give beer bitter flavors and aroma characteristics. The hop bittering compounds are classified into α-acids and soluble β-acids of lupulone, colupulone, and adlupulone. The α- and β-acids all have the basic alicyclic structure (2,4-cyclohexadiene-l-one) but the analogues differ in the nature of the acyl side chain (Stevens, 1987). The main bittering substances are the iso-α-acids formed during boiling of the wort. Many minor constituents are also present in the resins (Stevens, 1987).

Besides giving bitter flavor and aroma to beer, hop extracts have long been known to have antimicrobial activity. The β-resin component mixtures (lupulones) have been reported to have greater antimicrobial activity than the iso-α-resins (humulones) (Chin et al., 1949; Hough et al., 1957; Michener and Anderson, 1949; Shimwell, 1937a). The hop bitter acids inhibit gram-positive bacteria including species of *Bacillus*, *Micrococcus*, *Staphylococcus*, and others (Hough et al., 1957; Schmalreck et al., 1975). Inhibitory activity has also been reported for certain fungi (Engelson et al., 1980; Michener et al., 1948; Mizobuchi and Sato, 1985). The majority of the studies on the antimicrobial activities of hop extracts have evaluated the activities in culture media or in wort and beer but not in food.

We have been interested in whether hops could be used as an antimicrobial agent against bacterial pathogens that cause food poisoning. Although the incidence of listeriosis in the United States has declined (Tappero et al., 1995) since its clear recognition as a foodborne pathogen in the 1980s (Farber and Peterkin, 1991), the organism still is of concern as a potential pathogen, particularly for susceptible groups of humans. Recent foodborne outbreaks have occurred in Switzerland and France (Bula et al., 1995; Rocourt, 1995; Salvat et al., 1995). The organism is ubiquitous, associated with many raw foods (Beuchat, 1996; Farber and Peterkin, 1991; Ryser and Marth, 1991), and readily contaminates the food processing environment. Because of its ability to withstand sanitizing and food processing procedures, *L. monocytogenes* has found a place in the food industry as an indicator organism in evaluating the efficacy of sanitation procedures and good manufacturing practices.

Recent studies have shown that components of hop resins have antimicrobial

activity against *L. monocytogenes* in microbiological culture media (Barney et al., 1995; Millis and Schendel, 1994). However, the activity of antimicrobials in-vitro often does not accurately represent their efficacy in food. In this study, we have shown that hop resin extracts can inhibit *L. monocytogenes* in media and in certain foods.

2. Materials and methods

2.1. Organisms and growth in media

Strains of *L. monocytogenes* (strains Scott A, California strain, Ohio strain, V7 or LCDC 861—isolated from a listeriosis outbreak in coleslaw) were used in this study. Stock cultures were stored at −80°C in rich media containing 40% glycerol as a cryoprotectant. *L. monocytogenes* was grown at 37°C in trypticase soy broth (TSB; BBL Laboratories) or brain heart infusion broth (BHI; BBL Laboratories) (Larson et al., 1993; Premaratne et al., 1991). Growth was determined in culture media by determination of the optical density at 660 nm. One milligram of dry cell weight of *L. monocytogenes* corresponds to an optical density of 1.4 at 660 nm (Premaratne et al., 1991). Control experiments containing equivalent concentrations of Tween or ethanol were always conducted.

2.2. Hop preparations

Four hop acid extracts (HE; I–IV) were supplied by S.S. Steiner Inc., 655 Madison Avenue, New York, NY 10021. HE I was a preparation of iso-α-acids, 30% w/v, in aqueous suspension as the potassium salts. HE II contained 41% β-acids (an impure mixture of the various congeners), 12% α-acids, with the remainder consisting of desoxy-α-acids, hop oils, and hop waxes. HE III contained 29.7% colupulone, 65% β-acids (lupulone and adlupulon), 8% desoxy-α-acids, 7% H2O, and 0.6% iso-α-acids. HE IV contained post-β-acids (6% w/v). The post-β-acids consisted of the series of peaks that eluted after the β-acids (Moir and Smith, 1996). The compositions of the hop acid extracts were determined by reverse-phase high performance liquid chromatography (HPLC) on a C18 Nucleophil column (4.6 × 250 mm, 5 micron) (R.J. Smith, S.S. Steiner, Inc., Yakima, Washington, pers. commun.). The mobile phase consisted of 81.2% phosphoric acid, 0.1% EDTA, and the absorption of eluting compounds was determined at 270 nm.

2.3. Inhibition of L. monocytogenes in media

Inhibition studies were conducted mainly with *L. monocytogenes* Scott A since the four strains used in this study showed similar susceptibility in culture media. An overnight culture was inoculated into 10 ml of TSB or BHI broths containing a range of concentrations of the different hop extracts dissolved in ethanol or Tween

20 (Sigma). Tubes with equivalent quantities of ethanol or Tween 20 were also prepared as controls. The tubes were incubated statically at 37°C and growth monitored by measuring the optical density at 660 nm.

2.4. Inhibition of L. monocytogenes in foods

Commercial coleslaw (pH 3.8, 75.7% moisture, 0.23% titratable acidity) was obtained from a local market. HE III was dissolved in ethanol and added at various concentrations, and samples inoculated with a total of $\sim 10^5$ CFU/g of a mixture of *L. monocytogenes* strains Scott A, California, Ohio, V7, and LCDC 861 (isolated from a listeriosis outbreak in coleslaw (Schlech et al., 1983)). Incubation was carried out at 4 or 12°C, and survival of *L. monocytogenes* was determined at various intervals by diluting in 67 mM sodium phosphate, pH 6.6, and plating on MOX agar. The volume of hop extract dilutions added to each type of food was $\leq 1\%$ (w/v).

Commercial Grade A, lowfat, small curd cottage cheese (pH 4.5) was mixed with varying levels of HE II dissolved in Tween 20, inoculated with $\sim 10^3$ cfu/g of *L. monocytogenes* Scott A, and incubated at 4°C. Populations of *L. monocytogenes* were monitored by plating the cottage cheese periodically on MOX agar as described above for coleslaw.

Five gram wedges of Camembert cheese were mixed with different concentrations of HE III dissolved in ethanol. Samples were inoculated with $\sim 10^5$ cfu/g of *L. monocytogenes* Scott A, and homogenized to evenly distribute the hop extract and *L. monocytogenes*, and incubated at 4 or 12°C. Growth of *L. monocytogenes* was monitored by plating samples on MOX agar.

For determining the effect of hops on survival in milk, varying concentrations of HE III were added to skim, 2%, and whole milk, and the milk samples were inoculated with *L. monocytogenes* Scott A. The samples were incubated at 4°C, and the levels of *L. monocytogenes* determined by direct plating on modified Oxford agar.

2.5. Replications

All experiments were performed in duplicate. Each of the data points in the figures are the average of duplicate plates at two dilutions (total of four plates).

2.6. Chemical methods

Total acidity was determined by titration of a food sample to a phenolphthalein endpoint. The pH of the food and media was determined by a calibrated pH electrode. Total moisture was determined by drying to a consistent weight in a vacuum oven.

3. Results

3.1. Inhibition of L. monocytogenes by hop extracts in media

In the experimental conditions used, growth of *L. monocytogenes* usually reached a maximum after 22 to 26 h in TSB broth at 37°C. Table 1 shows inhibition by the hop extracts. The inhibition is expressed as percent growth after 24 h compared to the control samples containing equivalent concentration of Tween 20 or ethanol present after dissolution and dilution of the hop extracts.

HE I, consisting mainly of iso-α-acids, only moderately affected *L. monocytogenes* Scott A in TSB and 10–300 μg/ml were required for inhibition. The degree of inhibition at levels of HE I was not noticeably different for the two carriers (ethanol or Tween 20), except that 99% inhibition was observed with Tween 20 whereas inhibition in ethanol leveled off at ~66% inhibition.

Table 1
Inhibition of *L. monocytogenes* Scott A by hop extracts in TSB

Medium	Temperature (°C)	Hop extract	Carrier	Concentration (μg/ml)	% Inhibition (24 h)
TSB	37	HE I	Tween 20	10	15
				30	11
				100	61
				300	99
TSB	37	HE I	Ethanol	10	19
				30	25
				100	64
				300	66
TSB	37	HE II	Ethanol	0.3	12
				1	56
				3	62
				10	100
				30	100
				100	100
BHI	37	HE III	Ethanol	0.1	0
				0.3	31
				1	96
				3	98
				10	100
				30	100
TSB	37	HE IV	Ethanol	5	1
				10	4
				50	36
				100	47
				500	54
				1000	55

Percent inhibition is defiined as $A_{660\ nm}(+HE)/A_{660\ nm}$ (control containing equal concentration of carrier) × 100).

HE II, containing 41% (w/w) of a mixture of β-acids, showed strong inhibition of *L. monocytogenes* Scott A in TSB. Growth of *L. monocytogenes* was strongly inhibited by 10 μg/ml (Table 1). At the low concentration of 0.1 μg/ml HE II, cell growth was inhibited for 19 h, but after this period the cell population increased.

HE III, which contains $\sim 30\%$ colupulone and 65% β-acids (lupulone and adlupulone) was also effective in controlling *L. monocytogenes* in BHI or TSB broths at levels of ≥ 1 μg/ml. At this concentration of HE III, no growth was observed within 43 h, but growth did occur after this period. These results indicate that HE III was bactericidal at the higher concentrations tested, and was bacteriostatic at lower concentrations or that bacterial resistance was acquired at the lower concentrations.

HE IV, comprised of post-β-acids was poorly inhibitory to *L. monocytogenes*, and only slightly affected growth over the range of 50–500 μg/ml. Even at these concentrations growth eventually occurred, but at a slower rate than the control.

3.2. Inhibition of L. monocytogenes in food by hop extracts

The experiments in broth indicated that HE II and HE III had the strongest inhibitory activity against *L. monocytogenes*. In subsequent experiments, inhibition of *L. monocytogenes* by these hop extracts was evaluated in coleslaw, milk, cottage cheese, and Camembert cheese. These foods were chosen because they have been associated with outbreaks of listeriosis, or as vehicles for infection by *L. monocytogenes*.

3.3. Coleslaw

Coleslaw was an inhospitable environment for *L. monocytogenes* and the numbers of the pathogen decreased in all samplings and at both temperatures (Fig. 1A and 1B). At 4°C, populations decreased faster in the presence of 1000 μg/ml HE III (Fig. 1A). At 12°C, the numbers of *L. monocytogenes* decreased faster in the presence of 100 or 1000 μg/ml of HE III than in the controls (Fig. 1B).

3.4. Milk

L. monocytogenes grew in control samples of skim, 2% and whole milk at 4°C (Fig. 2A–C). In skim and 2% milk samples, 100 and 1000 μg/ml of HE III inhibited *L. monocytogenes* for 14 days. After this period, the populations of *L. monocytogenes* increased in the milk containing 100 μg/ml. HE III was not inhibitory in skim or 2% milk at < 100 μg/ml. This pattern was observed in repeated experiments. In whole milk, HE III was inhibitory at 1000 μg/ml but only slightly inhibitory at 100 μg/ml. Thus, HE III appeared to be more inhibitory in milk that contained less fat.

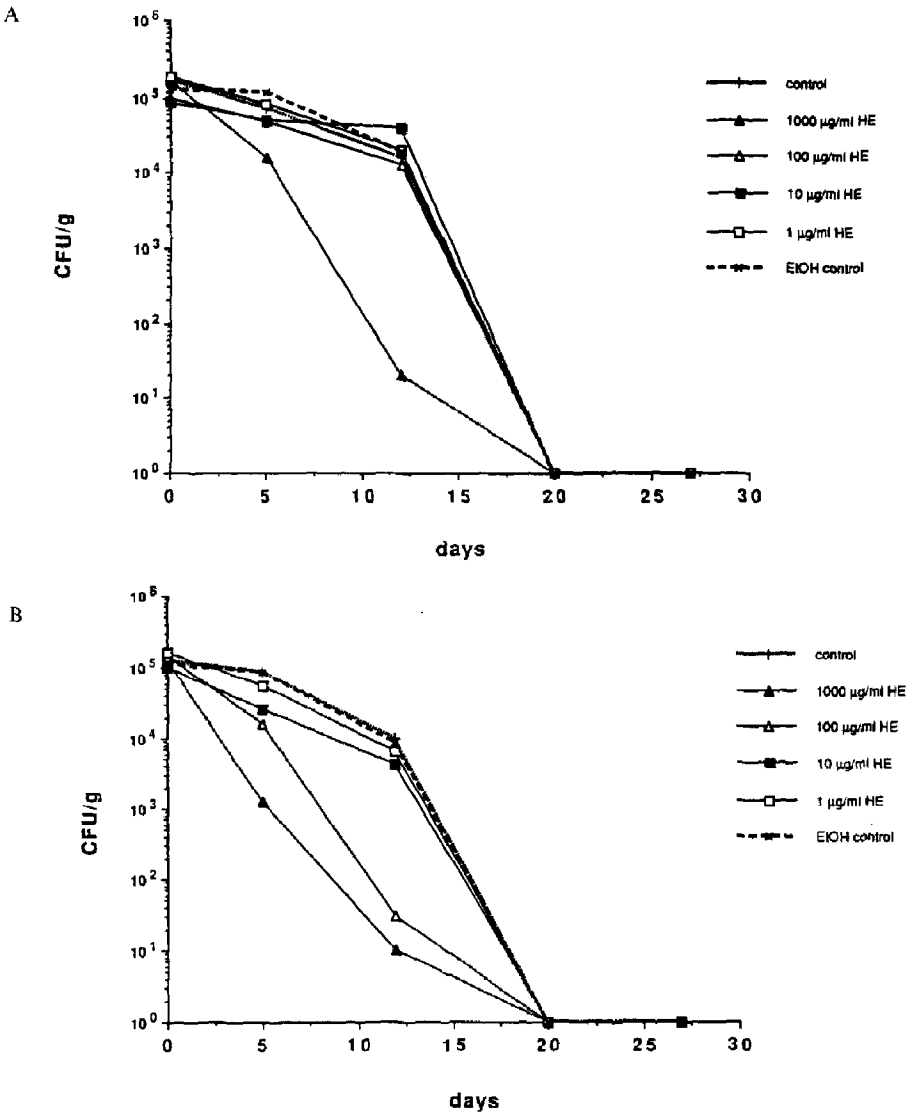

Fig. 1. Behavior at 4°C (panel A) and 12°C (panel B) of a mixture of *L. monocytogenes* strains (Scott A, California, Ohio, V7, and LCDC 861) by HE III in coleslaw containing hop extract.

3.5. Cottage cheese

L. monocytogenes grew in control incubations, or in cottage cheese containing ≤ 10 µg/g HE II at 4°C (Fig. 3). High levels of Tween 20 control (100 or 3000 µg/g) were inhibitory for 5–7 weeks, but populations increased after this time period. *L. monocytogenes* was not detected after 2 or 7 weeks of incubation in

cottage cheese containing 3000 or 1000 μg/g HE II respectively (Fig. 3). Populations also declined steadily in cottage cheese containing 100 μg/g HE II, but low levels ($\sim$ 10 cells/g) were detected at the termination of the incubation (11 weeks). These data indicate that HE II effectively inactivated *L. monocytogenes* in cottage cheese, but only at levels much higher than required for inhibition in media.

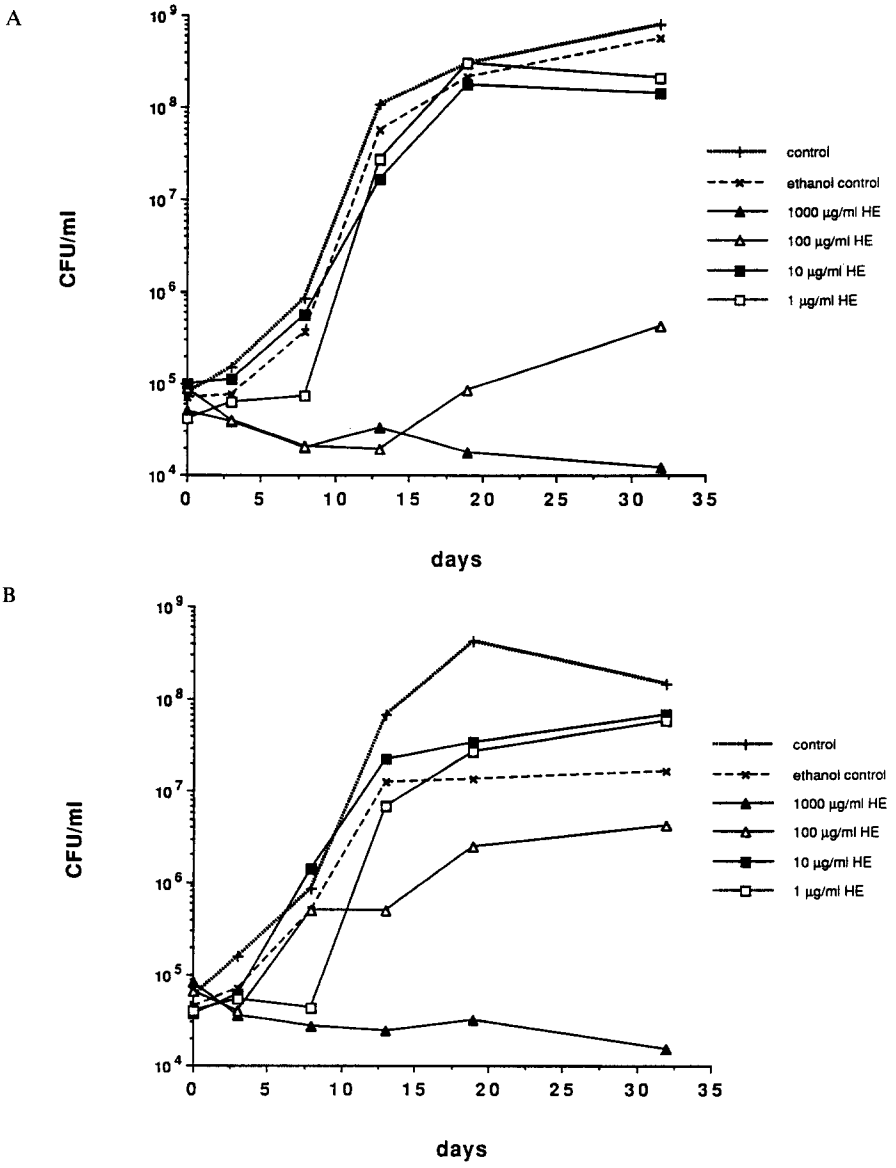

Fig. 2. Effect of HE II on behavior of *L. monocytogenes* Scott A in skim (A), 2% (B), and whole (C) milk at 4°C.

C

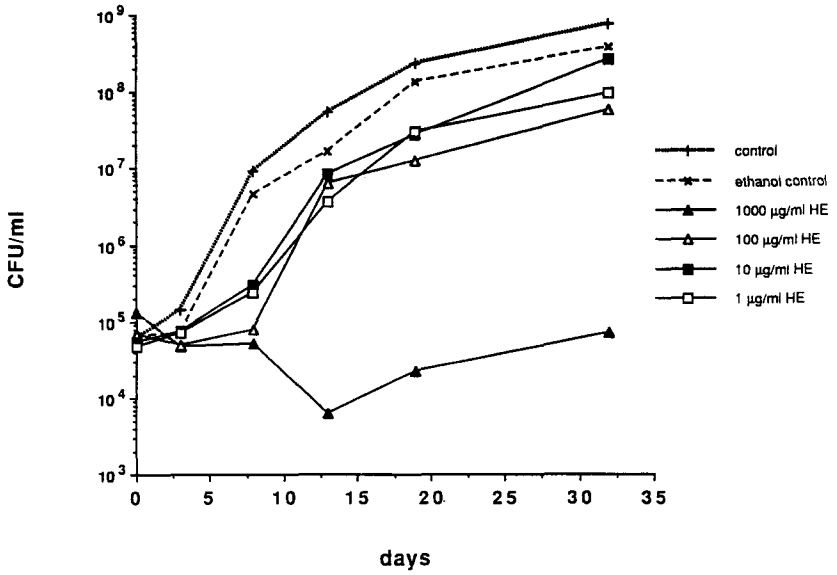

Fig. 2C.

3.6. Camembert cheese

At 4°C, levels of HE III as high as 10 000 µg/g did not inhibit *L. monocytogenes* relative to the control containing ethanol alone (Table 2A). At 12°C, *L. monocytogenes* grew to very high levels in Camembert and HE III was not inhibitory (Table 2B). These results indicate that HE III was ineffective towards *L. monocytogenes* in full-fat Camembert cheese.

4. Discussion

L. monocytogenes was sensitive to hop extracts in media but was much less sensitive in the foods tested. The results in media agree with data presented in two recently issued patents (Barney et al., 1995; Millis and Schendel, 1994). The investigators proposed that the hop components would also be effective in foods, but our study suggests that this will require much higher levels than are effective in media. These higher levels could impart undesirable flavors and aroma characteristics to the food.

In the present study, the degree of inhibition of *L. monocytogenes* was affected by characteristics of the food and the incubation conditions. The hops extracts were more effective in acidic foods (coleslaw and cottage cheese) than in foods with higher pH (milk and Camembert cheese). The pH has previously been reported as an important variable affecting activity of hops against gram-positive bacteria (Shimwell, 1937b; Simpson and Smith, 1992). Our laboratory previously reported

that monoglycerides are more effective against *L. monocytogenes* at pH 5 than at pH 6 (Wang and Johnson, 1992). The fat content also appeared to affect the efficacy of the hops extracts. It is likely that the reduced activity in Camembert cheese and in whole milk compared to skim and 2% milks was caused by sequestering of the hop acids in the lipid phase. We previously observed that other lipophilic antimicrobials, including monoglycerides, were less effective in whole than in 2% and skim milks against *L. monocytogenes* (Wang and Johnson, 1992; Wang et al., 1993). Lastly, the inhibitory activity of hops was generally increased at 4° compared to 12°C. At the lower temperature, the proportion of the hop extracts in the aqueous phase of the food may increase. Temperature would also affect the lipid content of the cytoplasmic membrane of *L. monocytogenes*, but the relation of cytoplasmic membrane lipid content to efficacy of lipophilic inhibitors is not clear.

The enrichment of lipid antimicrobials in the fatty phases of food presents a challenge in optimization of antimicrobial activity. Several studies have shown that the efficacy of various antimicrobials, including components of hop bitter resins, increases with hydrophobicity (Etoh et al., 1994; Hansch and Dunn, 1972; Schmalreck et al., 1975). This is in accordance with their proposed mode of action of perturbing membrane function (Teuber and Schmalreck, 1973). The efficacy of lipophilic antimicrobials in food would be enhanced by preventing their dissolution in the lipid phase.

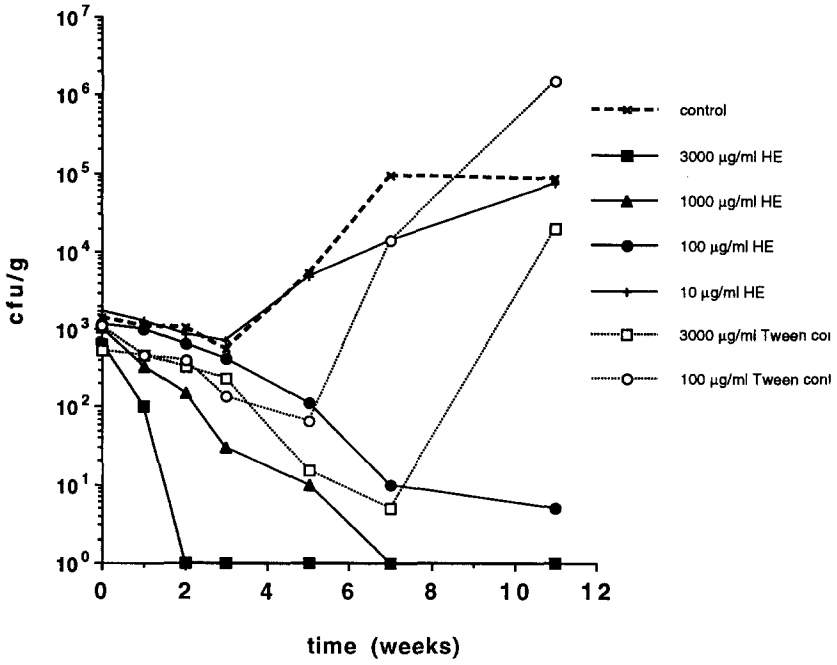

Fig. 3. Behavior of *L. monocytogenes* Scott A in cottage cheese containing HE II.

Table 2
A. Survival of *L. monocytogenes* in Camembert cheese with hop extract III at 4°C (log cfu/g)

	Day 0	Day 11	Day 18	Day 33
Control	5.48	6.39	7.41	7.02
Ethanol control	5.48	5.80	6.37	6.16
10 000 μg/ml HE	5.48	6.08	5.46	6.23
1000 μg/ml HE	5.48	5.74	6.41	5.56
100 μg/ml HE	5.48	6.45	6.04	5.68

B. Survival of *L. monocytogenes* in Camembert cheese with hop extract III at 12°C (log cfu/g)

	Day 0	Day 11	Day 18	Day 33
Control	5.48	9.01	8.85	9.02
Ethanol control	5.48	8.34	8.34	8.18
10 000 μg/ml HE	5.48	8.11	7.04	7.28
1000 μg/ml HE	5.48	8.36	8.29	8.41
100 μg/ml HE	5.48	8.85	8.97	8.72

Another potential drawback to the use of hops and other related compounds is the ability of microorganisms to gain resistance by mutation, by physiological adaptation, or by detoxification of the inhibitors. In the present study, we observed that *L. monocytogenes* was often inhibited transiently and that on continued incubation growth was observed. Other genera of bacteria have also been reported to gain resistance to hop acids (Fernandez and Simpson, 1993; Haas and Barsoumian, 1994; Haas and Herman, 1976).

In conclusion, we have found that hop extracts are inhibitory to *L. monocytogenes* in media and in certain foods. The activity we observed in low-fat and moderately acidic foods at low temperature suggests that hop β-acids could be used as barriers in low-fat or refrigerated foods to prevent growth of *L. monocytogenes* when processing or intrinsic protection is inadequate.

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